Research Report

Four-Color Flow Cytometric Detection of Retrovirally Expressed Red, Yellow, Green, and Cyan Fluorescent Proteins

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ABSTRACT

Flow cytometric procedures are described to detect a "humanized" version of a new red fluorescent protein (DsRed) from the coral Discosoma sp. in conjunction with various combinations of three Aequorea victoria green fluorescent protein (GFP) variants—EYFP, EGFP, and ECFP. In spite of overlapping emission spectra, the combination of DsRed with EYFP, EGFP, and ECFP generated fluorescence signals that could be electronically compensated in real time using dual-laser excitation at 458 and 568 nm. Resolution of fluorescence signals from DsRed, EYFP, and EGFP was also readily achieved by single-laser excitation at 488 nm. Since many flow cytometers are equipped with an argon-ion laser that can be tuned to 488 nm, the DsRed/EYFP/ EGFP combination is expected to have broad utility for facile monitoring of gene transfer and expression in mammalian cells. The dual-laser technique is applicable for use on flow cytometers equipped with tunable multiline argon-ion and krypton-ion lasers, providing the framework for studies requiring simultaneous analysis of four fluorescent gene products within living cells.

INTRODUCTION

The cloning of the green fluorescent protein (GFP) gene from the luminescent jellyfish *Aequorea victoria* has led to its widespread use as a marker for gene expression in various biological systems (5–7,22). Site-directed mutations have yielded variants amenable to detection by both fluorescence microscopy and flow cytometry in mammalian cells (9,11). Due to overlapping signals, fluorescence microscopy is used primarily for the detection of individual fluorescent proteins, whereas flow cytometry is employed for the simultaneous analysis of multiple fluorescent proteins (1–3,17,23,24). Using the latter methodology, successful separation of the emission wavelengths of up to three GFP variants has been reported (4,25). In particular, a flow cytometric protocol was recently described in which single-laser excitation at 458 nm allowed simultaneous analysis of yellow (EYFP), green (EGFP), and cyan (ECFP) emitters in transiently transfected cells (4).

The recent isolation of a red fluorescent protein gene (drFP583) from *Discosoma* sp., a nonbioluminescent reef coral (Phylum *Cnidaria*: Class *Anthozoa*), promises to complement techniques using the *Aequorea* GFP variants by expanding the range of the spectrum available for detection by more than 50 nm (19). The subsequent introduction of a human codon-optimized version of the drFP583 gene, DsRed, should further extend the usefulness of this reporter in mammalian systems (Clontech Laboratories, Palo Alto, CA, USA). Visualization of DsRed by fluorescence microscopy in triple-labeling experiments with EYFP and ECFP has been documented by the manufacturer (8); however, a transient transfection assay was used, and the procedure involved fixing the cells and capturing images of the individual fluorescent proteins using separate filter sets. Flow cytometric analysis of DsRed fluorescence in transiently transfected cells was also presented (8). However, it was not known whether the DsRed signal is bright enough to permit detection from stably introduced transgenes in viable mammalian cells using flow cytometric methodology nor whether the spectral properties of this red fluorescent protein are suitable for simultaneous multicolor analyses with GFP variants.

The ability to simultaneously detect four fluorescent proteins in living cells will provide the opportunity to efficiently introduce four (or more) different genes of interest into cell populations using polycistronic retroviral vectors for genetic modification and lineage marking applications (6,10,15, 16,20,21). To this end, the present study was undertaken to evaluate the feasibility of flow cytometric analysis of DsRed in combination with EYFP, EGFP, and ECFP using dual-laser excitation at 458 and 568 nm. Realizing that not all flow cytometers are equipped with expensive tunable multiline lasers, we also assessed an alternative three-color detection protocol for DsRed, EYFP, and EGFP that is applicable for use on instruments having single-laser excitation at 488 nm.

MATERIALS AND METHODS

Retroviral Vectors and Cell Line Generation

The pDsRed1-1, pEYFP-N1, pEGFP-1, and pECFP plasmids encoding DsRed, EYFP, EGFP, and ECFP, respectively, were obtained from Clon-

tech Laboratories. The MGIN retroviral vector contains the EGFP gene expressed from the murine stem cell virus (MSCV) long terminal repeat on a bicistronic transcript that also contains a downstream neomycin resistance (Neo[r]) gene under the control of an encephalomyocarditis virus internal ribosome entry site (6). The MRIN, MYIN, and MCIN retroviral vectors containing the DsRed, EYFP, and ECFP genes were similarly constructed by inserting the respective fluorescent protein genes into the same MSCV-based Neo[r] vector backbone (13,14). Stable cell lines expressing the MRIN, MYIN, MGIN, and MCIN retroviral vectors were generated by transduction of GP+E-86 ecotropic packaging cells followed by G418 selection (400 µg/mL Geneticin®; Life Technologies, Rockville, MD, USA) and/or cell sorting as previously described (12). GP+E-86 is a derivative of the murine embryonic fibroblast cell line, NIH3T3, and was originally obtained from Dr. Arthur Bank (Columbia University, New York, NY, USA) (18).
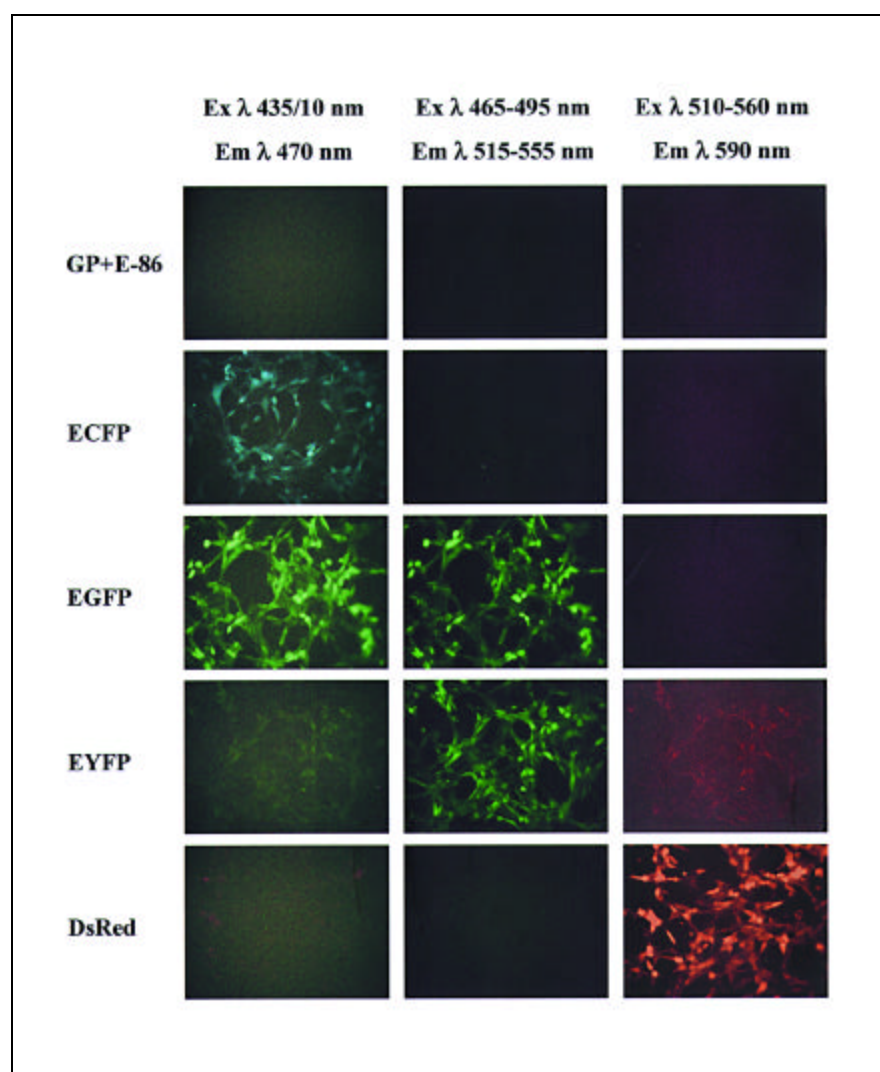
Fluorescence Microscopy

Fluorescent proteins were visualized individually under a Nikon Eclipse TE300 inverted fluorescence microscope (Nikon, Melville, NY, USA) equipped with a 100-W mercury arc lamp and a Catseye Digital Camera (Sony Electronics, San Jose, CA, USA). DsRed was detected with a G-2A filter set (EX 510-560, DM 575, BA 590), EYFP, and EGFP with a B-2E/C filter set (EX 465-495, DM 505, BA 515-555), and ECFP with a BV-1A filter set (EX 435/10, DM 455, BA 470).

Flow Cytometry

Transduced cells were analyzed on a FACSVantage™ SE (BD Biosciences, San Jose, CA, USA) equipped with Coherent I-90 argon-ion and Coherent I-302C krypton-ion lasers (Coherent, Santa Clara, CA, USA). For dual-laser analysis, the primary laser (I-90) was tuned to 458 nm and the second laser (I-302C) was tuned to 568 nm. The three-beam separation option allowed the krypton-ion laser beam to be directed along the third laser pathway, maximizing spatial separation of the two beams and minimizing both laser noise and signal crosstalk between the signals. For single-laser analysis, the primary laser (I-90) was tuned to 488 nm. Argon-ion 458- and 488-nm laser line power outputs were set at 100 and 70 mW, respectively, and the krypton-ion 568-nm laser line power output was set at 35 mW. Data were acquired using CELLQuest™ (BD Biosciences). The OmniCompensation option (BD Biosciences) allowed real-time intra- and inter-laser compensations. For comparison purposes, software-based compensation (WinList; Verity Software House, Topsham, ME, USA) was applied to data acquired without hardware compensation in some experiments.

To detect all four fluorescent proteins, EYFP, EGFP, and ECFP were excited at 458 nm, while DsRed was excited at 568 nm. The ECFP signal was split off from the EYFP/EGFP signals using a 500-nm longpass dichroic filter and collected with a 485/22 bandpass (BP) filter. The EYFP and EGFP signals were separated with a 525-nm shortpass dichroic filter and collected

Figure 1. Fluorescence microscopic analysis of four fluorescent proteins using three standard filter sets. GP+E-86 cells individually expressing DsRed, EYFP, EGFP, and ECFP were imaged using each filter set as indicated. The BV-1A filter set (EX 435/10, DM 455, BA 470) used to image the ECFP signal also captured EGFP fluorescence and, to a lesser extent, EYFP fluorescence. The B-2E/C filter set (EX 465–495, DM 505, BA 515–555) used for fluorescein isothiocyanate-like emission could not distinguish between the EGFP and EYFP signals. The G-2A filter set (EX 510–560, DM 575, BA 590) used for phycoerythrin-like emission detected the DsRed signal and the EYFP signal. Images were overexposed to demonstrate spectral overlap.

Research Report

with a 550/30 BP filter and a 510/20 BP filter, respectively. A 568-nm restriction band filter was placed in front of the 550/30 BP filter to block off stray laser light. The DsRed signal was collected with a 610/30 BP filter with a sharp cutoff at 595 nm.

Three of the four fluorescent proteins—DsRed, EYFP, and EGFP—could be excited at 488 nm. The DsRed signal was split off from the EYFP/EGFP signals using a 560-nm shortpass dichroic filter and collected with a 585/42 BP filter. The EYFP and EGFP signals were detected as described above. All optical filters were supplied by BD Biosciences or purchased from Omega Optical (Brattleboro, VT, USA) or Chroma Technology (Brattleboro, VT, USA).

To determine whether DsRed could be used in conjunction with surface-labeling fluorochromes, GP+E-86 cells expressing DsRed were incubated with a biotin-conjugated anti-mouse CD90 monoclonal antibody and either streptavidin-PE-Cy5 or streptavidin-PE-Cy7 in a second step. The biotin-conjugated anti-mouse CD90 monoclonal antibody and the streptavidin-PE-Cy5 conjugate were purchased from BD Biosciences. The streptavidin-PE-Cy7 conjugate was purchased from Caltag Laboratories (Burlingame, CA, USA). The antibody and secondary reagents were used at concentrations recommended by the manufacturers.

RESULTS AND DISCUSSION

Spectral Overlap of Fluorescent Proteins Illustrated by Fluorescence Microscopy

Permanent cell lines stably expressing DsRed, EYFP, EGFP, ECFP, and DsRed plus EGFP were generated by retroviral-mediated gene transfer (see Materials and Methods). No toxicity due to the sustained expression of DsRed or any of the GFP variants was observed. Before flow cytometric analysis, cells expressing individual fluorescent protein genes were evaluated by fluorescence microscopy using standard filter sets that closely matched the excitation and emission maxima of the respective fluorescent proteins (Table 1). All four fluorescent proteins were easily

Table 1. Spectral Characteristics of the Red, Yellow, Green, and Cyan Fluorescent Proteins[a]

Protein	Color	Excitation Maximum (nm)	Emission Maximum (nm)
DsRed	red	558	583
EYFP	yellow	514	527
EGFP	green	489	508
ECFP	cyan	434	477

[a]Data from Reference 8.

Table 2. Instrument Settings for Analysis of Four Fluorescent Proteins Expressed by GP+E-86 Cells Using Dual-Laser (458 and 568 nm) Flow Cytometry[a]

Parameter	Fluorescent Protein	PMT Voltage	Compensation			
			(-%FL1)	(-%FL2)	(-%FL3)	(-%FL4)
FL1	EGFP	438	–	67.6%	61.3%	0%
FL2	EYFP	753	49.2%	–	6%	0%
FL3	ECFP	380	18.3%	0%	–	0%
FL4	DsRed	483	0%	0%	0%	–

[a]Each voltage setting was adjusted with non-transduced negative control cells and cells expressing a particular fluorescent protein to position the fluorescence signal on scale. Compensation was set using CELLQuest and the OmniCompensation circuitry. Parameter settings were fine-tuned to achieve a balance of compensations.

visualized, but spectral overlap was prominent, especially among the GFP variants (Figure 1). Considerable fluorescence from EGFP and, to a lesser extent, EYFP was observed when the cells were viewed under the filter set used for ECFP detection. In addition, EYFP and EGFP signals were both detected with the filter set designed for fluorescein isothiocyanate fluorescence. As expected from their spectral characteristics, some of the EYFP signal was detected with the filter set used for DsRed fluorescence, whereas no signals were detected from EGFP and ECFP.

Flow Cytometric Detection of DsRed and Three GFP Variants

In flow cytometry, the problem of spectral overlap can be dealt with by employing the appropriate optical filter configuration and real-time electronic (hardware) or post-analysis software-based compensation (1,2,4,17,24,25). Because simultaneous flow cytometric

analysis of the three GFP variants under study was recently demonstrated using single-laser excitation at 458 nm (4), we reasoned that the resolution of fluorescence signals from all four fluorescent proteins might be attainable by exciting EYFP, EGFP, and ECFP at 458 nm and DsRed at a wavelength close to its excitation maximum (558 nm). Accordingly, cells expressing individual fluorescent proteins were analyzed on the flow cytometer using dual-laser excitation at 458 and 568 nm. This latter excitation wavelength was chosen because it is available on the tunable multiline krypton-ion laser. At 458 nm, all four fluorescent proteins are excited, approximately equally well for EGFP and ECFP (60% of maximum absorbance), less well for DsRed (<20% of maximum absorbance), and the least well for EYFP (<10% of maximum absorbance) (8). The resolution of EYFP, EGFP, and ECFP fluorescence was achieved by intra-laser compensation (Table 2). Po-

Table 3. Instrument Settings for Analysis of Three Fluorescent Proteins Expressed by GP+E-86 Cells Using Single-Laser (488 nm) Flow Cytometry[a]

| Parameter	Fluorescent Protein	PMT Voltage	Compensation (-%FL1)	Compensation (-%FL2)	Compensation (-%FL3)
FL1	E G F P	457	–	52.8%	0%
FL2	EYFP	619	78.8%	–	1.6%
FL3	DsRed	381	0%	67.2%	–

[a]Parameter settings were adjusted as described in Table 2 for the dual-laser analysis.

tential interference of these signals with DsRed detection could have been dealt with by inter-laser compensation. However, the latter process was obviated by selective use of a 610/30 BP filter, which excluded signals from these three fluorescent proteins from the DsRed fluorescence channel. Conversely, because of its red-shifted emission spectrum, DsRed fluorescence generated from either 458- or 568-nm excitation did not interfere with the signals of the other three fluorescent proteins. At 568-nm excitation, no signals were elicited from EYFP, EGFP, or ECFP. Under these conditions, the signals from all four fluorescent proteins were successfully resolved in real time by careful pair-wise compensations between detectors (Figure 2).

Three-Color Fluorescent Protein Detection Using Single-Laser (488-nm) Excitation

DsRed has a minor excitation peak around 488 nm and can be excited reasonably efficiently at this wavelength (40% of maximum absorbance) (8). Since many flow cytometers are
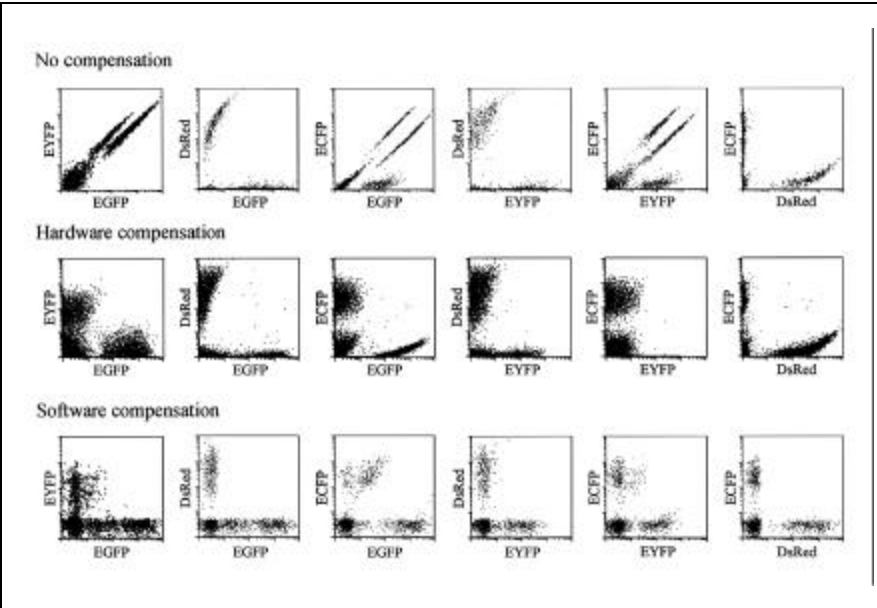
Figure 2. Flow cytometric analysis of four fluorescent proteins using excitation wavelengths at 458 and 568 nm. Resolution of fluorescence signals from DsRed, EYFP, EGFP, and ECFP was obtained in a mixture of GP+E-86 cells expressing the individual proteins (together with non-transduced negative control cells). Top row: uncompensated data. Middle row: data generated in real time with electronic (hardware) compensation (CELLQuest and OmniCompensation; see Table 2 for instrument settings). Note that the compensation required to completely eliminate spectral overlap of intense fluorescence signals resulted in an apparent overcompensation of cells expressing lower levels of the fluorescent proteins (see Reference 4). Bottom row: the results obtained by applying post-analysis software compensation (WinList) to the uncompensated data are presented for comparison purposes.

Research Report

equipped with an argon-ion laser that can be tuned to 488 nm, the possibility of analyzing the DsRed/EYFP/EGFP combination using single-laser excitation at this wavelength was evaluated. As shown in Figure 3, fluorescence signals from the three fluorescent proteins were readily detected and could be distinguished in real time under the conditions employed (Table 3).
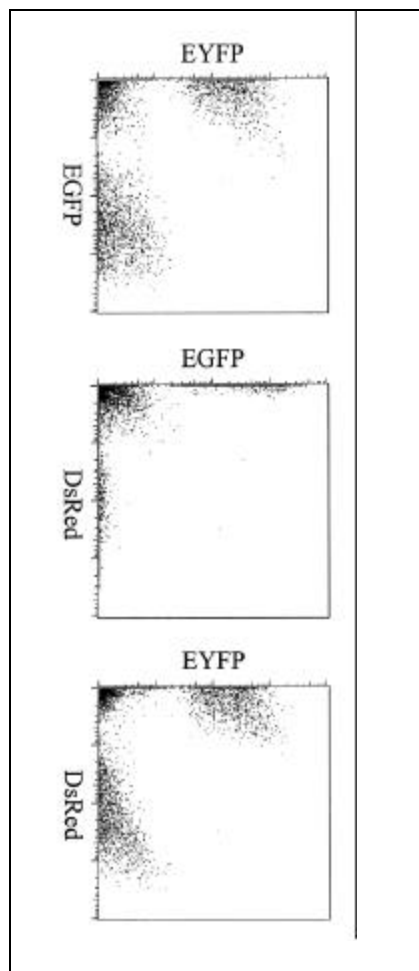
Simultaneous Analysis of DsRed in Conjunction with EGFP, PE-Cy5, or PE-Cy7

Simultaneous detection and discrimination of DsRed and EGFP fluorescence using dual-laser excitation was demonstrated using populations of cells expressing either or both of these fluorescent proteins (Figure 4A). Cells simultaneously expressing DsRed and EGFP could also be easily discriminated from cells expressing the individual fluorescent proteins using the single-laser excitation procedure (Figure 4B). Based on these latter results, we predicted that, for cytometers equipped with four detectors off of a primary 488-nm laser beam, cells marked with the DsRed/EYFP/EGFP combination could also be labeled with fluorochromes emitting beyond 600 or 650 nm, depending on whether a 585/42 BP filter or a 610/30 BP filter was used for DsRed detection. Possible candidates included PE-Texas Red, PE-Cy5, PerCP-Cy5.5, and PE-Cy7. Analysis of DsRed-expressing GP+E-86 cells labeled with anti-CD90-PE-Cy5 or anti-CD90-PE-Cy7 confirmed the ability to simultaneously detect DsRed and a surface antigen with these monoclonal antibody-fluorochrome conjugates using this protocol (Figure 4, C–F).

CONCLUDING REMARKS

We report the development of two flow cytometry methods to detect the newly described DsRed and up to three GFP variants. In one method, we adapted an optical filter configuration that was recently introduced to simultaneously detect EYFP, EGFP, and ECFP and employed dual-laser line excitation at 458 and 568 nm to demonstrate the feasibility of identifying distinct cell populations stably marked with these three GFP variants plus DsRed. Flow cytometric data on simultaneous detection of DsRed and EGFP at the single-cell level was also presented. Simultaneous detection of the EYFP/EGFP, EYFP/ECFP, or EGFP/ECFP combinations in individual cells was previously shown using 458-nm excitation (4). More recently, we have generated permanent cell lines expressing DsRed plus EYFP, EGFP, and ECFP (unpublished data). Collectively, these observations establish that it is possible with the dual-laser technique described here

Figure 3. Flow cytometric analysis of three fluorescent proteins using single-laser excitation at 488 nm. Real-time resolution of fluorescence signals from DsRed, EYFP, and EGFP in a mixture of GP+E-86 cells expressing the individual proteins (together with non-transduced negative control cells). See Table 3 for instrument settings.
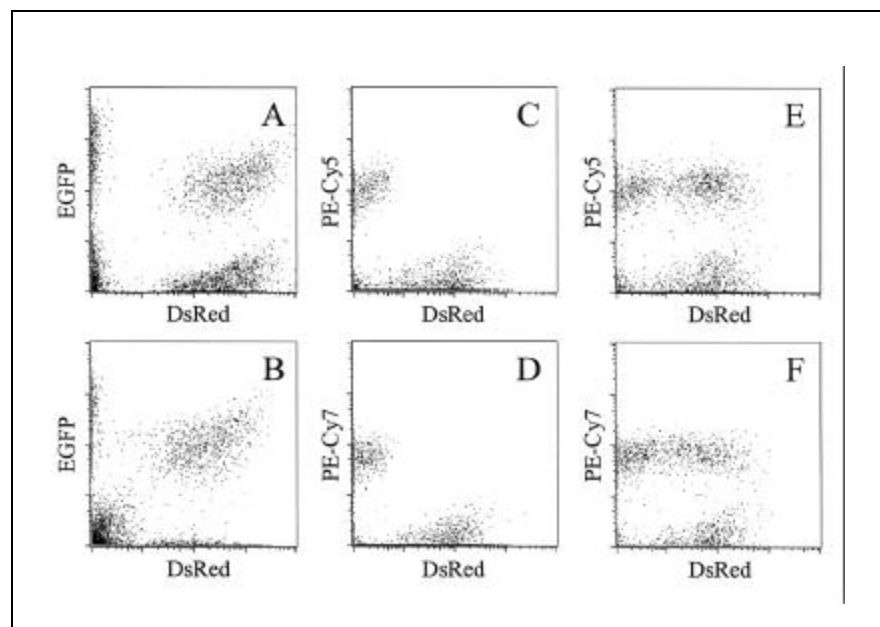
Figure 4. Simultaneous analysis of DsRed in conjunction with EGFP or a surface antigen. Plot A shows simultaneous detection of DsRed and EGFP in a mixture of GP+E-86 cells expressing either or both fluorescent proteins (together with non-transduced negative control cells) using dual-laser (458 and 568 nm) excitation. Plot B shows similar detection using single-laser (488 nm) excitation. Plots C and D indicate complete resolution of DsRed fluorescence and a surface-labeling fluorochrome (PE-Cy5 or PE-Cy7) using single-laser (488-nm) excitation. A 610 nm shortpass dichroic filter split the signals detected with the 585/42 BP filter for DsRed and the signals detected with the 675/20 BP filter for PE-Cy5. For PE-Cy7, a 745 nm EFLP filter was used. Plot E shows simultaneous detection of DsRed and PE-Cy5 in a mixture of singly and doubly labeled GP+E-86 cells. Plot F shows simultaneous detection of DsRed and PE-Cy7 in a mixture of singly and doubly labeled GP+E-86 cells.

Research Report

to directly detect and viably sort sub-populations of cells simultaneously expressing DsRed and various combinations of these three GFP variants for mammalian studies of gene function and cell tracking (6,10,15,16,20,21).

We also introduced a new three-color detection scheme involving DsRed, EYFP, and EGFP that uses single-laser excitation at 488 nm and provided flow cytometric data showing simultaneous detection of DsRed and EGFP, DsRed and PE-Cy5, or DsRed and PE-Cy7 at the single-cell level. It is anticipated that this DsRed-based protocol will have broader utility in triple-labeling experiments with fluorescent proteins than three-color flow cytometric methods that include ECFP or the blue-shifted variant of GFP because the latter require excitation with laser lines that are not available on standard flow cytometers (4,25).

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